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ScienceDirect
Veterinary Immunology and Immunopathology 114 (2006) 259–272
Veterinary immunology and immunopathology
www.elsevier.com/locate/vetimm

Changes in immune-related gene expression and intestinal lymphocyte subpopulations following *Eimeria maxima* infection of chickens

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Received 21 April 2006; received in revised form 10 August 2006; accepted 11 August 2006

Abstract

Coccidiosis, a major intestinal parasitic disease of poultry, induces a cell-mediated immune response against the etiologic agent of the disease, *Eimeria*. In the current study, the expression levels of gene transcripts encoding pro-inflammatory, Th1, and Th2 cytokines, as well as chemokines were measured in intestinal intraepithelial lymphocytes (IELs) after *Eimeria maxima* infection. In addition, changes in IEL numbers were quantified following *E. maxima* infection. Transcripts of the pro-inflammatory and Th1 cytokines IFN-γ, IL-1β, IL-6, IL-12, IL-15, IL-17, and IL-18 were increased 66- to 8×10^7-fold following primary parasite infection. Similarly, mRNA levels of the Th2 cytokines IL-3, IL-10, IL-13, and GM-CSF were up-regulated 34- to 8800-fold, and the chemokines IL-8, lymphotactin, MIF, and K203 were increased 42- to 1756-fold. In contrast, IFN-α, TGF-β4, and K60 transcripts showed no increased expression, and only the level of the Th2 cytokine IL-13 was increased following secondary *E. maxima* infection. Increases in intestinal T cell subpopulations following *E. maxima* infection also were detected. $CD3^+$, $CD4^+$, and $CD8^+$ cells were significantly increased at days 8, 6, and 7 post-primary infection, respectively, but only $CD4^+$ cells remained elevated following secondary infection. $TCR1^+$ cells exhibited a biphasic pattern following primary infection, whereas $TCR2^+$ cells displayed a single peak in levels. Taken together, these data indicate a global chicken intestinal immune response is produced following experimental *Eimeria* infection involving multiple cytokines, chemokines, and T cell subsets.

Keywords: *Eimeria*; Cytokines; Chemokines; T cells; Quantitative RT-PCR

1. Introduction

Coccidiosis is a major parasitic disease responsible for substantial economic losses in the poultry industry due to mortality, malabsorption, inefficient feed utilization, and impaired growth rate of broilers and reduced egg production by layers (Lillehoj et al., 2004). A variety of methods are used commercially to overcome this disease, including use of antibiotics, selection of disease resistant chicken strains, and manipulation of chicken immunity (Lillehoj, 1994; Caron et al., 1997; Pinard-Van

Abbreviations: DPI, days post-primary infection; DSI, days post-secondary infection; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GM-CSF, granulocyte monocyte-colony stimulating factor; IELs, intraepithelial lymphocytes; iNOS, inducible nitric oxide synthase; MIF, migration inhibition factor; MIP-1β, macrophage inflammatory protein-1β; MyD88, myeloid differentiation factor 88; T-bet, T-box expressed in T cells; TGF-β4, transforming growth factor-β4; TRAF-5, TNF receptor-associated factor-5

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doi:10.1016/j.vetimm.2006.08.006

Der Laan et al., 1998; Yun et al., 2000b; Dalloul and Lillehoj, 2006). Many basic research laboratories worldwide are currently investigating the latter option, and it is becoming increasingly clear that chicken immune responses to coccidial infection are quite complex. Animals infected with *Eimeria* spp. produce parasite-specific antibodies in both the circulation and mucosal secretions. However, it appears that antibody-mediated responses play a minor role in protective immunity against coccidiosis. Instead, cell-mediated immunity constitutes the major host response conferring resistance to parasite infection. T lymphocytes, natural killer (NK) cells, and macrophages are involved in the avian cellular immune response to *Eimeria* infection (Lillehoj and Trout, 1996).

Until recently, only a limited number of immune regulator and effector genes and their encoded polypeptides were identified in avians due to the low level of sequence homologies with their mammalian counterparts. However, with the advent of the chicken genome project, many new genes encoding avian immune-related molecules have been identified (Hughes and Bumstead, 2000; Schneider et al., 2000; Sick et al., 2000; Min and Lillehoj, 2002; Avery et al., 2004; Degen et al., 2004; Min and Lillehoj, 2004; Rothwell et al., 2004; Hwang et al., 2005; Kaiser et al., 2005; Read et al., 2005; Hong et al., 2006). While some of these genes have been characterized and shown to be similar to their mammalian homologues, the roles of the encoded polypeptides in avian immunity and the host response to pathogen infections requires further study (Lillehoj et al., 2004). In particular, future analysis of avian cytokines and chemokines will provide valuable new information on protective immunity to infectious diseases currently plaguing the poultry industry, including not only coccidiosis, but also influenza, salmonellosis, and necrotic enteritis (Lillehoj and Bacon, 1991; Rothwell et al., 1995; Laurent et al., 2001; Sadeyen et al., 2004; Swaggerty et al., 2004; Withanage et al., 2004, 2005; Dalloul et al., 2007).

Cytokines are essential effector molecules of innate and adaptive immunity against pathogenic microorganisms. Current dogma holds that cytokines are classified by the Th1–Th2 paradigm according to their role in the immune response (Mosmann et al., 1986; Mosmann and Coffman, 1989; Degen et al., 2005). As in mammals, host inflammation and innate immunity in chickens are mediated by Th1 or Th1-related cytokines, including interferon (IFN)-α, -β, and -γ (Sekellick et al., 1994; Digby and Lowenthal, 1995; Sick et al., 1996), interleukin (IL)-1β (Weining et al., 1998), IL-2 (Sundick and Gill-Dixon, 1997), IL-6 (Schneider et al., 2001), IL-10 (Rothwell et al., 2004), IL-12 (Degen et al., 2004), IL-15 (Lillehoj et al., 2001), IL-16 (Min and Lillehoj, 2004), IL-17 (Min and Lillehoj, 2002), and IL-18 (Schneider et al., 2000). Some of these cytokines are expressed through the T-bet (T-box expressed in T cells) transcription factor (Hong et al., unpublished observations). In addition, other molecules such as myeloid differentiation factor 88 (MyD88), TNF receptor associated factor-5 (TRAF-5), and inducible nitric oxide synthase (iNOS) are involved in the Th1 inflammatory response to avian pathogens (Lin et al., 1996; Caldwell et al., 2005).

Th2 or Th2-related molecules, classically defined as driving humoral immunity, produce a distinct panel of cytokines, in particular IL-3, IL-4, IL-5, IL-13, granulocyte macrophage-colony stimulating factor (GM-CSF), and transforming growth factor (TGF)-β4 (Jakowlew et al., 1988; Avery et al., 2004), some of which are expressed through the GATA-3 transcription factor (Ishihara et al., 1995). A third group of immune effector molecules, the chemokines, are important mediators of cell migration during inflammation and in normal leukocyte trafficking. Chemokines are grouped into four structural families (CXC, CC, XC, CX3C) characterized by the position of their amino-terminal cysteine residues (Zlotnik and Yoshie, 2000) and more than 20 chemokines have been identified in the chicken genome (Hwang et al., 2005; DeVries et al., 2006). CXC, or α chemokines, in chickens are IL-8 (Kaiser et al., 1999), K60 (Sick et al., 2000), and SDF-1 (Read et al., 2005). Chicken CC (or β) chemokines include macrophage inflammatory protein (MIP)-1β (Petrenko et al., 1995; Hughes and Bumstead, 1999), migration inhibitory factor (MIF) (Wistow et al., 1993), and K203 (Sick et al., 2000). Chicken lymphotactin (Rossi et al., 1999; Hughes and Bumstead, 2000) is a C (or γ) chemokine.

The goal of the current study was to comprehensively investigate the dynamics of cytokine and chemokine gene expression and intestinal lymphocyte subpopulations following experimental infection of chickens with *E. maxima* in order to better characterize the development of protective immunity against coccidiosis.

2. Materials and methods

2.1. Chickens, experimental Eimeria infections, and collection of intestinal IELs

Fertilized eggs of specific pathogen-free chickens were obtained from SPAFAS (Charles River Laboratories, Wilmington, MA) and hatched at the Animal and Natural Resources Institute, USDA (Beltsville, MD).

Chickens were kept in wire cages with feed and water provided *ad libitum*, and orally inoculated with 1×10^4 sporulated oocysts of *E. maxima* Tyson at 3 weeks of age as described (Min et al., 2001). Secondary infection with 2×10^4 oocysts was performed at day 14 post-primary infection. Day 0 non-infected samples at primary (3-week old) and secondary (5-week old) infections were used as controls. At 1 day intervals following primary or secondary infections, the intestinal jejunum was removed from five chickens in each group, cut longitudinally, and washed three times with ice-cold Hank's balanced salt solution (HBSS) containing 100 units/ml of penicillin and 100 μg/ml of streptomycin (Sigma, St. Louis, MO). The mucosal layer was carefully scraped away using a surgical scalpel, the tissue was washed several times with HBSS containing 0.5 mM EDTA and 5% fetal calf serum (FCS) and incubated for 20 min at 37 °C with constant swirling. Cells released into the supernatant were pooled, passed through nylon wool (Robbins Scientific, Sunnyvale, CA) to remove dead cells and cell aggregates and washed twice with HBSS. IELs were purified on a discontinuous percoll density gradient by centrifugation at $600 \times g$ for 25 min at 24 °C (Chai and Lillehoj, 1988). All experiments were approved by the Animal and Natural Resources Institute IACUC.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was extracted from IELs using TRIzol (Invitrogen, Carlsbad, CA) as described (Min et al., 2001). Five microgram of total RNA were treated with 1.0 unit of DNase I and 1.0 μl of 10× reaction buffer (Sigma), incubated for 15 min at room temperature, 1.0 μl of stop solution was added to inactivate DNase I, and the mixture was heated at 70 °C for 10 min. RNA was reverse-transcribed using StrataScript first-strand synthesis system (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. Briefly, 5.0 μg of RNA was combined with 10× first strand buffer, 1.0 μl of oligo(dT) primer (5 μg/μl), 0.8 μl of dNTP mix (25 mM of each dNTP), and RNase-free water to total volume 19 μl. The mixture was incubated at 65 °C for 5 min, cooled to room temperature, 50 units of StrataScript reverse transcriptase was added, the mixture was incubated at 42 °C for 1 h, and the reaction stopped by heating at 70 °C for 5 min.

2.3. Quantitative real-time RT-PCR

Quantitative RT-PCR oligonucleotide primers for chicken cytokines, chemokines, and GAPDH control are listed in Table 1. Amplification and detection were carried out using equivalent amounts of total RNA from IELs using the Mx3000P system and Brilliant SYBR Green QPCR master mix (Stratagene). Standard curves were generated using $\log_{10}$ diluted standard RNA and levels of individual transcripts were normalized to those of GAPDH analyzed by the Q-gene program (Muller et al., 2002). Each analysis was performed in triplicate. To normalize RNA levels between samples within an experiment, the mean threshold cycle (C_t) values for the amplification products were calculated by pooling values from all samples in that experiment.

2.4. Antibodies and flow cytometric analysis

Single cell suspensions of IELs were prepared from infected and non-infected chickens (three per group) as described (Yun et al., 2000a) and resuspended in HBSS without phenol red supplemented with 3% FCS and 0.01% sodium azide (FCA buffer). The cells were incubated for 30 min on ice with mouse monoclonal antibodies (mAbs) specific for chicken macrophages (K1), CD3, CD4, CD8, γδ-T cell receptor (TCR1), or αβ-T cell receptor (TCR2) (Lillehoj et al., 1988). As a negative control, HB2, an antibody specific for human T cells (American Type Culture Collection) was included and antibody against C6B12, a chicken MHC class I antigen, was used as a positive control. Following incubation, the cells were washed three times with FCA buffer, incubated with 50 μl of fluorescein isothiocyanate-labeled goat anti-mouse IgG secondary antibody (Sigma) for 30 min on ice, and fluorescence measured in an EPICS XL-MCL flow cytometer (Coulter, Hialeah, FL) with 1×10^4 viable cells. The percentage of cells bound by each mAb was calculated with respect to those bound by the control C6B12 mAb.

2.5. Statistical analysis

Mean ± S.E. values for each group ($N = 3$ or 5) were calculated, differences between groups were analyzed by the Dunnet multiple comparison test using InStat® software (Graphpad, San Diego, CA), and considered significant at $p < 0.01$ or 0.001.

3. Results

3.1. Pro-inflammatory cytokine response during E. maxima infection

E. maxima invade and develop within enterocytes of the upper-to-mid region of the small intestine,

Table 1
Sequence of the oligonucleotide primers used in quantitative RT-PCR

RNA target	Primer sequences		Size for PCR product (bp)	Accession no.
	Forward	Reverse		
GAPDH	5′-GGTGGTGCTAAGCGTGTTAT-3′	5′-ACCTCTGTCATCTCTCCACA-3′	264	K01458
IFN-γ	5′-AGCTGACGGTGGACCTATTATT-3′	5′-GGCTTTGCGCTGGATTC-3′	259	Y07922
IFN-α	5′-GACATCCTTCAGCATCTCTTCA-3′	5′-AGGCGCTGTAATCGTTGTCT-3′	238	AB021154
IL-1β	5′-TGGGCATCAAGGGCTACA-3′	5′-TCGGGTTGGTTGGTGATG-3′	244	Y15006
IL-2	5′-TCTGGGACCACTGTATGCTCT-3′	5′-ACACCAGTGGGAAACAGTATCA-3′	256	AF000631
IL-3	5′-CTCTGCCTGCTGCTGTCC-3′	5′-TTATCTGCTTTTTGCTGCTTTC-3′	238	AJ621740
IL-4	5′-ACCCAGGGCATCCAGAAG-3′	5′-CAGTGCCGGCAAGAAGTT-3′	258	AJ621735
IL-6	5′-CAAGGTGACGGAGGAGGAC-3′	5′-TGGCGAGGAGGGATTTCT-3′	254	AJ309540
IL-8	5′-GGCTTGCTAGGGGAAATGA-3′	5′-AGCTGACTCTGACTAGGAAACTGT-3′	200	AJ009800
IL-10	5′-CGGGAGCTGAGGGTGAA-3′	5′-GTGAAGAAGCGGTGACAGC-3′	272	AJ621614
IL-12p40	5′-AGACTCCAATGGGCAAATGA-3′	5′-CTCTTCGGCAAATGGACAGT-3′	274	NM_213571
IL-13	5′-CCAGGGCATCCAGAAGC-3′	5′-CAGTGCCGGCAAGAAGTT-3′	256	AJ621735
IL-15	5′-TCTGTTCTTCTGTTCTGAGTGATG-3′	5′-AGTGATTTGCTTCTGTCTTTGGTA-3′	243	AF139097
IL-16	5′-TCCCTCTGCAAAATGGTCA-3′	5′-TCGCGATCTCAGGTTGTGT-3′	271	AJ508678
IL-17	5′-CTCCGATCCCTTATTCTCCTC-3′	5′-AAGCGGTTGTGGTCCTCAT-3′	292	AJ493595
IL-18	5′-GGAATGCGATGCCTTTTG-3′	5′-ATTTTCCCATGCTCTTTCTCA-3′	264	AJ277865
TGF-β4	5′-CGGGACGGATGAGAAGAAC-3′	5′-CGGCCCACGTAGTAAATGAT-3′	258	M31160
GM-CSF	5′-CGCCCACCACAACATACTC-3′	5′-ACGATTCCGCTTTCTTCCT-3′	202	AJ621740
MIF	5′-GCAGCCTCTACAGCATTGG-3′	5′-TCTAACGGGCAGCACGAG-3′	229	M95776
K60	5′-ATTTCCTCCTGCCTCCTACA-3′	5′-GTGACTGGCAAAAATGACTCC-3′	228	AF277660
K203	5′-ACCACGAGCTCCTGACACA-3′	5′-TTAAATGCCCTCCCTACCAC-3′	300	Y18692
MIP-1β	5′-GTGCCCTCATGCTGGTGT-3′	5′-GGTTGGATGCGGATTATTTC-3′	285	L34553
Lymphotactin	5′-GGATTTAAGGGTGAACAGTAGATG-3′	5′-TAGAAATAGAAAGCCCGAGGAT-3′	254	AF006742
TRAF-5	5′-TGATTATCCCATGCCTTGTCT-3′	5′-CTCTGCTAGCTGCTGGATTTTA-3′	283	NM_204219
MyD88	5′-TCCCGGCGGTAGACAGC-3′	5′-ACGACCACCATCCTCCGACACCTT-3′	274	NM_001030962
iNOS	5′-TGGGTGGAAGCCGAAATA-3′	5′-GTACCAGCCGTTGAAAGGAC-3′	241	U46504
T-bet	5′-GGGAACCGCCTCTACCTG-3′	5′-AGTGATGTCGGCGTTCTGG-3′	288	CB016768

eliciting antibody and cell-mediated immune responses (Lillehoj and Trout, 1996; Yun et al., 2000b; Lillehoj et al., 2004). Our previous studies demonstrated that several pro-inflammatory cytokines, such as IFN-γ and IL-17, were produced in response to experimental *Eimeria* infections (Lillehoj and Choi, 1998; Min et al., 2001). However, the detailed kinetics of expression of these cytokines and others that have since been described, have not been determined. Therefore, we determined the expression levels of intestinal IEL gene transcripts encoding IFN-α, IL-1β, IL-6, and IL-17 at daily intervals for 10 days following primary *E. maxima* infection and for 8 days following secondary infection. As shown in Fig. 1, three of the cytokines (IL-1β, IL-6, and IL-17) showed significantly higher mRNA levels following primary inoculation compared with non-infected animals (day 0), ranging from 66- to 1650-fold increases ($p < 0.01$). The maximum fold changes for particular transcripts compared with non-infected day 0 values are indicated in parentheses in each figure. In contrast, the levels of IFN-α were unchanged following primary infection and transcripts for all four cytokines were unchanged or only slightly changed following secondary infection.

3.2. Th1 and Th2 cytokine responses following E. maxima infection

Analysis of the Th1 and Th1-related cytokines IFN-γ, IL-2, IL-10, IL-12, IL-15, IL-16, and IL-18, as well as transcripts encoding MyD88, TRAF-5, iNOS, and T-bet, revealed that only IFN-γ, IL-10, IL-12, IL-15, and IL-18 were highly increased in intestinal IELs following *E. maxima* primary infection (Fig. 2). Interestingly, IL-2 mRNA levels were significantly down-regulated during this time. As with the pro-inflammatory response, few of the Th1 cytokines or other transcripts examined were changed following secondary parasite infection. For mRNAs encoding the Th2 and anti-inflammatory cytokines IL-3, IL-4, IL-13, and GM-CSF, increases ranging from 40- to 3.7×10^3-fold were observed following primary infection except for TGF-β4 (Fig. 3). Unique to all the molecules examined in this study, only IL-13 transcript levels were increased following secondary parasite infection.

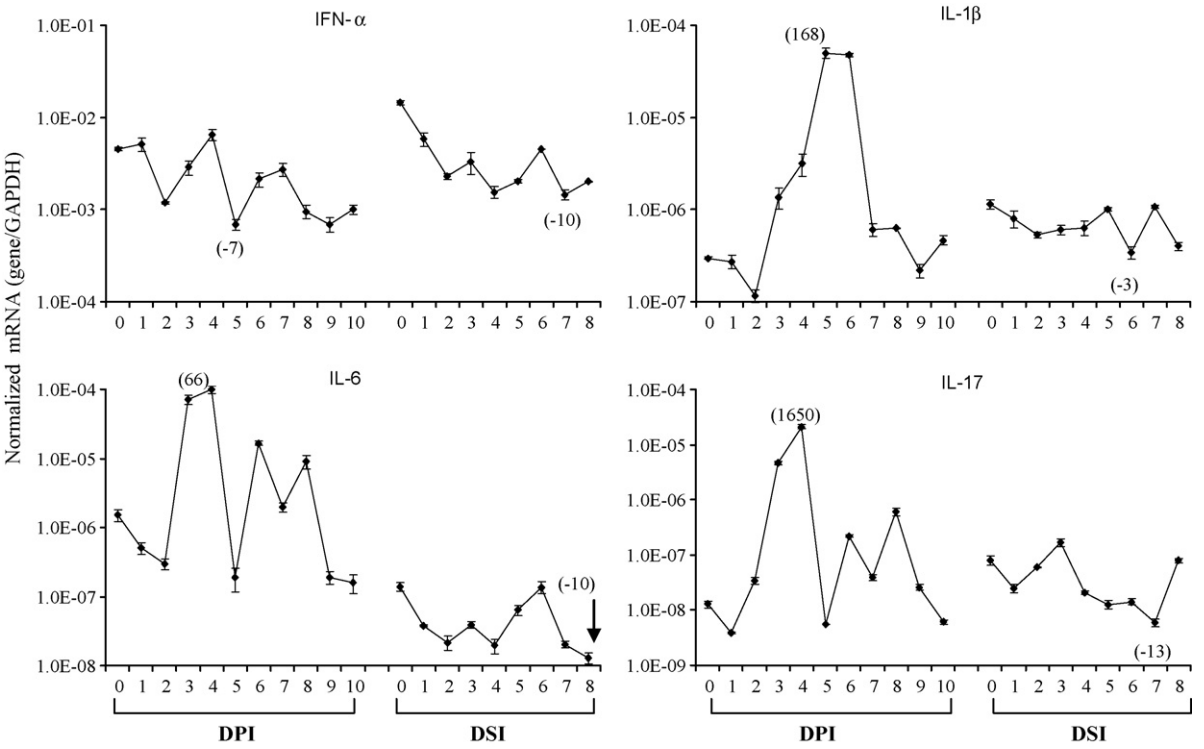

Fig. 1. Quantitative RT-PCR determination of pro-inflammatory cytokine mRNA levels after *E. maxima* infection. Chickens were non-infected (day 0) or orally infected with 1×10^4 oocysts of *E. maxima* on day 0 and reinfected with 2×10^4 oocysts on day 14 post-primary infection. Intestinal IELs were isolated from the jejunum at the indicated times post-infection and mRNA levels determined. Data are expressed as mean ± S.E. values of normalized mRNA to GAPDH mRNA of triplicate determinations with pooled samples from five chickens. Ratios between gene expression at particular days, when compared with non-infected values (day 0 at DPI and DSI), are indicated in parentheses as positive (increased) or negative (decreased) values, statistically significant at $p < 0.01$. DPI, days post-primary infection; DSI, days post-secondary infection.

3.3. Expression of chemokines during E. maxima infection

Chemokines are important mediators of cell migration during inflammation and in normal leukocyte trafficking of monocytes, eosinophils, basophils and T lymphocytes. Among the six chicken chemokines analyzed, IL-8, K203, MIF, MIP-1β, and lymphotactin were significantly up-regulated following primary *E. maxima* infection, whereas K60 was marginally up-regulated following primary and secondary infections (Fig. 4).

3.4. Changes in intestinal lymphocyte subpopulations during E. maxima infection

Alterations of intestinal lymphocyte subpopulations following *E. maxima* primary and secondary infections were investigated to better understand the nature of protective immunity during coccidiosis. The numbers of cells expressing the K1, CD3, CD4, CD8, γδ-TCR (TCR1), and αβ-TCR (TCR2) surface antigens were analyzed by fluorescence-activated flow cytometry and expressed as the ratio of positive cells from infected vs. non-infected chickens (Fig. 5). All subpopulations examined exhibited significantly increased levels following primary parasite infection compared with non-infected animals. $CD3^+$, $CD4^+$, and $CD8^+$ cells were significantly increased at days 8, 6, and 7 post-primary infection, respectively, while $CD4^+$ cells were increased following both inoculations. $TCR1^+$ cells exhibited a biphasic pattern following primary infection, whereas $TCR2^+$ cells displayed a single peak in levels.

4. Discussion

In this study, we describe the cytokine and chemokine responses and dynamics of intestinal lymphocyte subpopulations at various time-points following *E. maxima* infection. In general, expression of pro-inflammatory, Th1 and Th2 cytokines, and chemokines were significantly increased after primary infection, but relatively unchanged following secondary

infection. Similarly, most intestinal lymphocyte subsets were increased after primary parasite inoculation. Only $CD4^+$ cells were increased during both primary and secondary infections.

The effect of IFN-α on *Eimeria* parasites was previously investigated using recombinant chicken IFN-α, but these studies failed to demonstrate any effects on the HD11, DU24, CHCC-OU2, or LMH cell lines (Heriveau et al., 2000). Moreover, *in vivo* injection of a cDNA encoding chicken IFN-α did not influence the efficacy of a recombinant coccidiosis vaccine (3-1E) in stimulating immunity to *E. acervulina* (Min et al.,

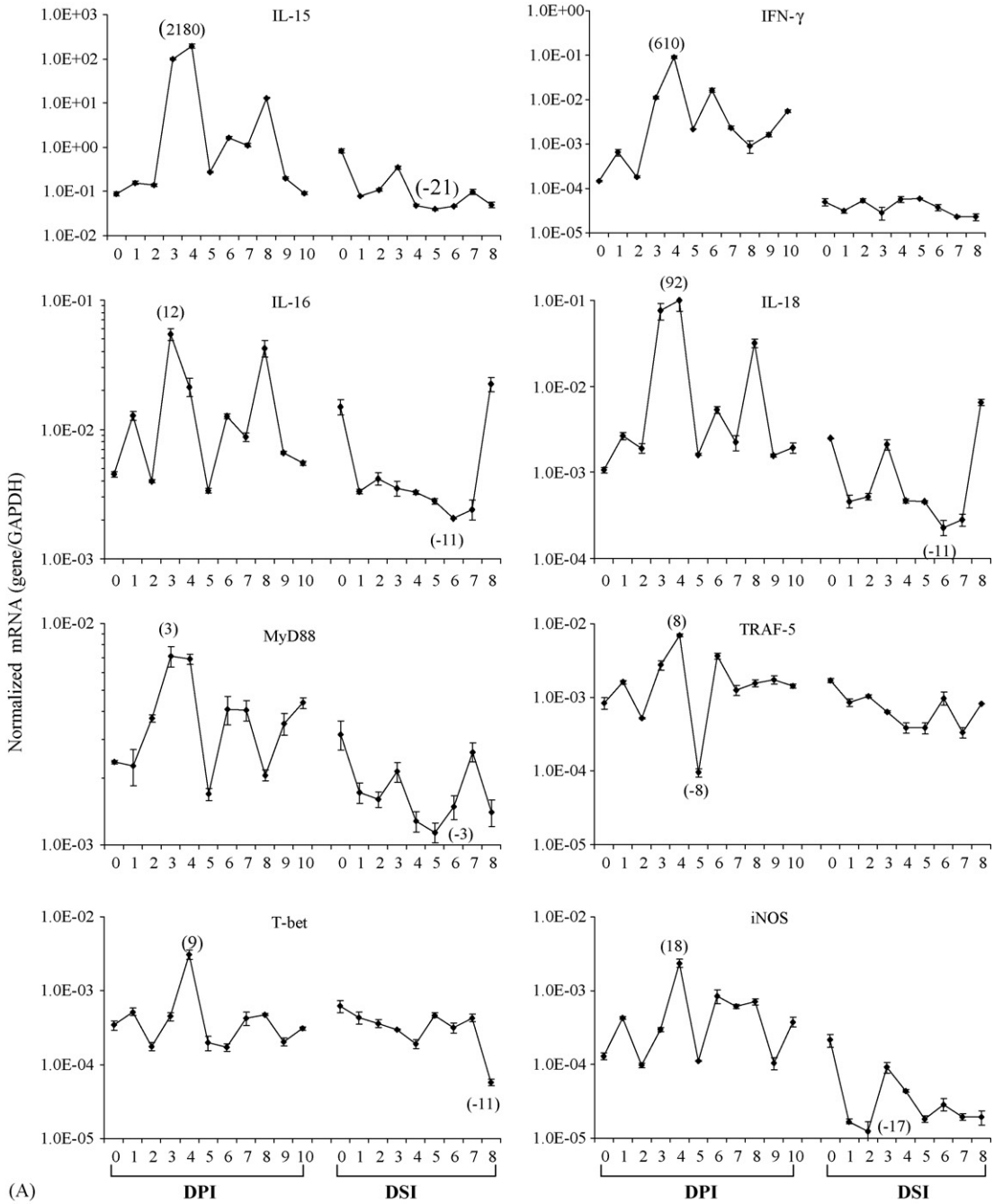

Fig. 2. Quantitative RT-PCR determination of Th1 cytokine and other inflammatory effector molecule mRNA levels after *E. maxima* infection. Chickens were infected with *E. maxima* and mRNA levels determined as in Fig. 1.

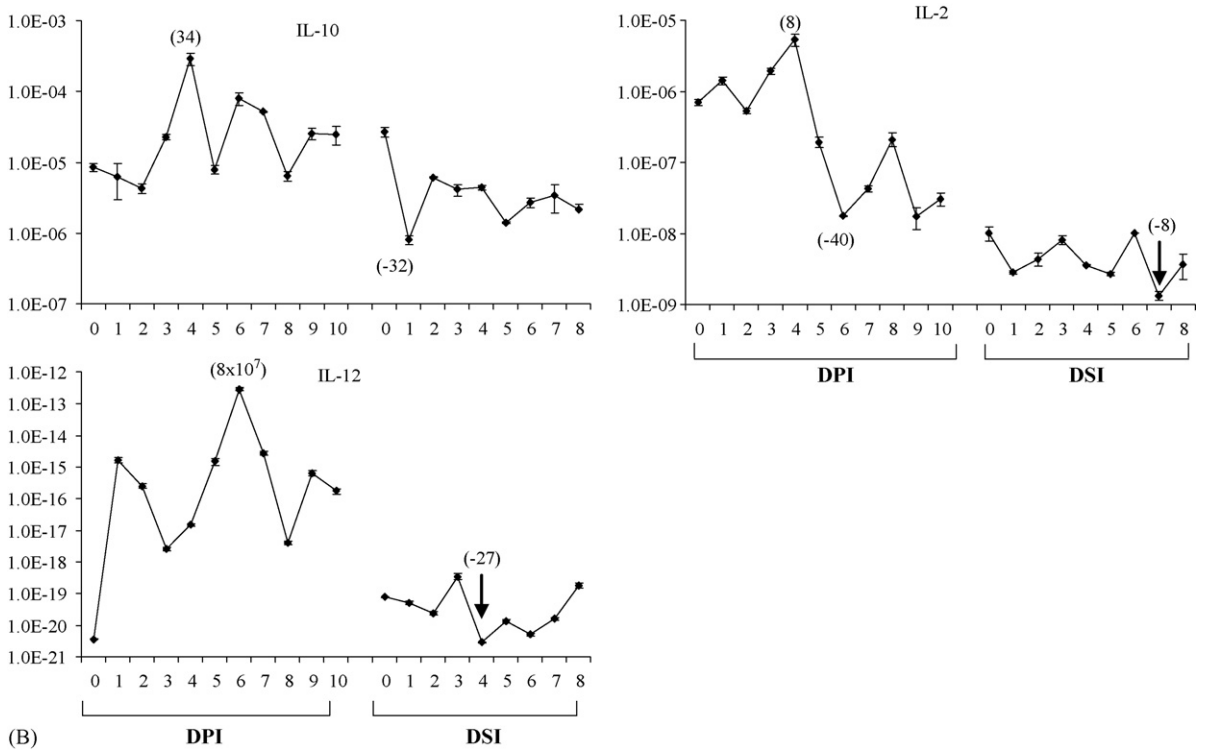

Fig. 2. (*Continued*).

2001). Our current observations corroborate these prior results since we observed that IFN-α mRNA levels were not affected during *E. maxima* experimental infection. Hence, unlike viral infections, our results suggest that type I interferons (α/β) do not mediate the early innate immune response to *Eimeria* infection.

IL-1β is a powerful pro-inflammatory cytokine secreted by many different cell types, with stimulated macrophages being the major producer. Lymphocytes from *Eimeria*-infected chickens produced a higher level of IL-1β protein compared with cells from non-infected birds (Byrnes et al., 1993), and quantitative RT-PCR measurements of IL-1β mRNA production demonstrated 27–80-fold increased transcript levels at day 7 post-primary infection with *E. maxima* and *E. tenella* (Laurent et al., 2001) or *Salmonella* infection (Withanage et al., 2004). Similarly, we observed increased IL-1β mRNA levels at day 4 following primary parasite infections. IL-6 and IL-8 are usually indicative of the initiation of an acute-phase response and were up-regulated following primary infection with *E. maxima* with similar kinetics (Figs. 1 and 4). Also, it has been proposed that increased expression of IL-6 may create a population of heterophils, avian equivalents of mammalian neutrophils, more capable of responding to and eliminating pathogens (Swaggerty et al., 2004). IL-17 is produced by activated and memory T cells and, like IL-1β, induces the production of other pro-inflammatory cytokines, such as TNF-α and IL-1β. The current results demonstrated that the 1650-fold up-regulation of IL-17 transcripts following *E. maxima* infection was the greatest among all of the pro-inflammatory cytokines examined. It should be noted, however, that IL-17 transcripts were not detected by Northern blot analysis at 0, 4, 8, 12, and 24 h following *in vitro* treatment of chicken intestinal IELs (Min and Lillehoj, 2002).

IFN-γ is a major cytokine mediating resistance to many different parasites or *Salmonella* (Sadeyen et al., 2004), and has been shown to inhibit *E. tenella* development *in vitro* (Lillehoj and Choi, 1998), and decrease oocyst production and improve body weight gain following *E. acervulina* infection (Lowenthal et al., 1997; Lillehoj and Choi, 1998). Using a quantitative RT-PCR assay, increased IFN-γ mRNA expression was observed in lymphocytes isolated from the intestinal ceca of *E. tenella*-infected SC strain chickens, and the selective depletion of $CD4^+$, but not $CD8^+$ cells, reduced IFN-γ production (Yun et al., 2000a). The current studies support these prior observations since we noted significant up-regulation

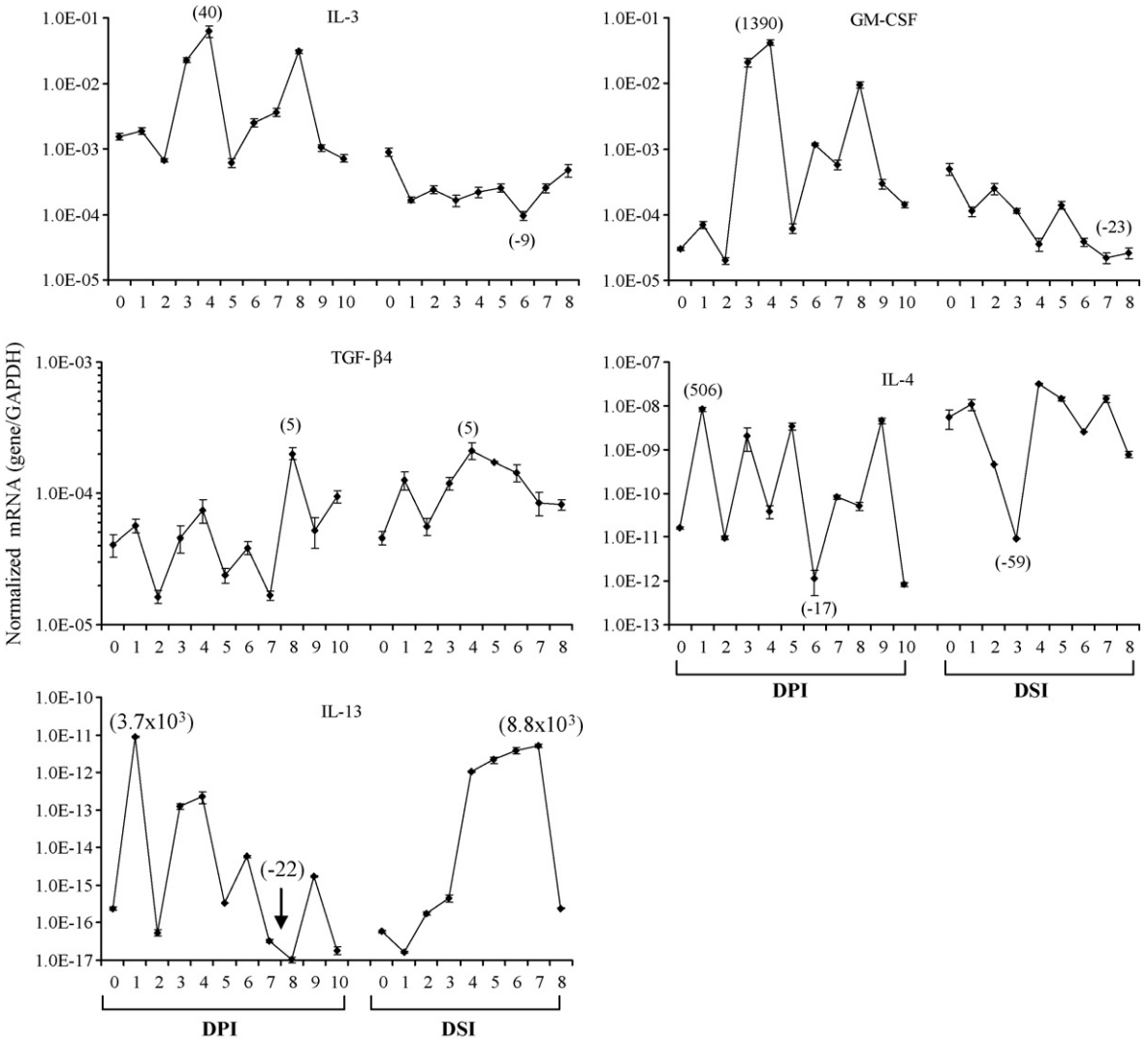

Fig. 3. Quantitative RT-PCR determination of Th2, anti-inflammatory (TGF-β4) and GM-CSF cytokine mRNA levels after *E. maxima* infection. Chickens were infected with *E. maxima* and mRNA levels determined as in Fig. 1.

of IFN-γ expression in the jejunum of *E. maxima*-infected chickens, probably due in large part to the recruitment and stimulation of $CD4^+$ and $TCR2^+$ cells.

IL-10 is a pleiotropic cytokine produced mainly by activated macrophages or $CD4^+$ and $CD8^+$ T cells (Groux and Powrie, 1999) and is involved in the control of innate immune reactions and cell-mediated immunity by inhibiting the production of IL-12 by activated macrophages and dendritic cells. Rothwell et al. (2004) reported that chicken IL-10 mRNA expression occurred mainly in the bursa of Fabricius and cecal tonsils, as well as by LPS-stimulated monocyte-derived macrophages. Moreover, expression of IL-10 and IFN-γ mRNAs at 6 and 9 dpi was significantly increased. In our own studies, IL-10, IL-12, and IFN-γ mRNA expression was robustly increased at days 4–6 (Fig. 2), and in $CD4^+$ and $CD8^+$ subpopulations (Fig. 5). Therefore, we speculate that IL-10 may play a crucial role in Th1–Th2 balance by preventing the development of Th1 cytokines as described by Rothwell et al. (2004).

IL-15 is produced by mononuclear phagocytes and other cell types in response to infection by viruses or parasites, LPS, and other signals. Chicken IL-15 is structurally homologous to IL-2 and stimulates the proliferation of NK cells (Sundick and Gill-Dixon, 1997; Lillehoj et al., 2001). After primary and secondary infections with *E. acervulina*, a significant enhancement in the levels of IL-2 transcripts was observed in the spleen and intestine (Choi and Lillehoj,

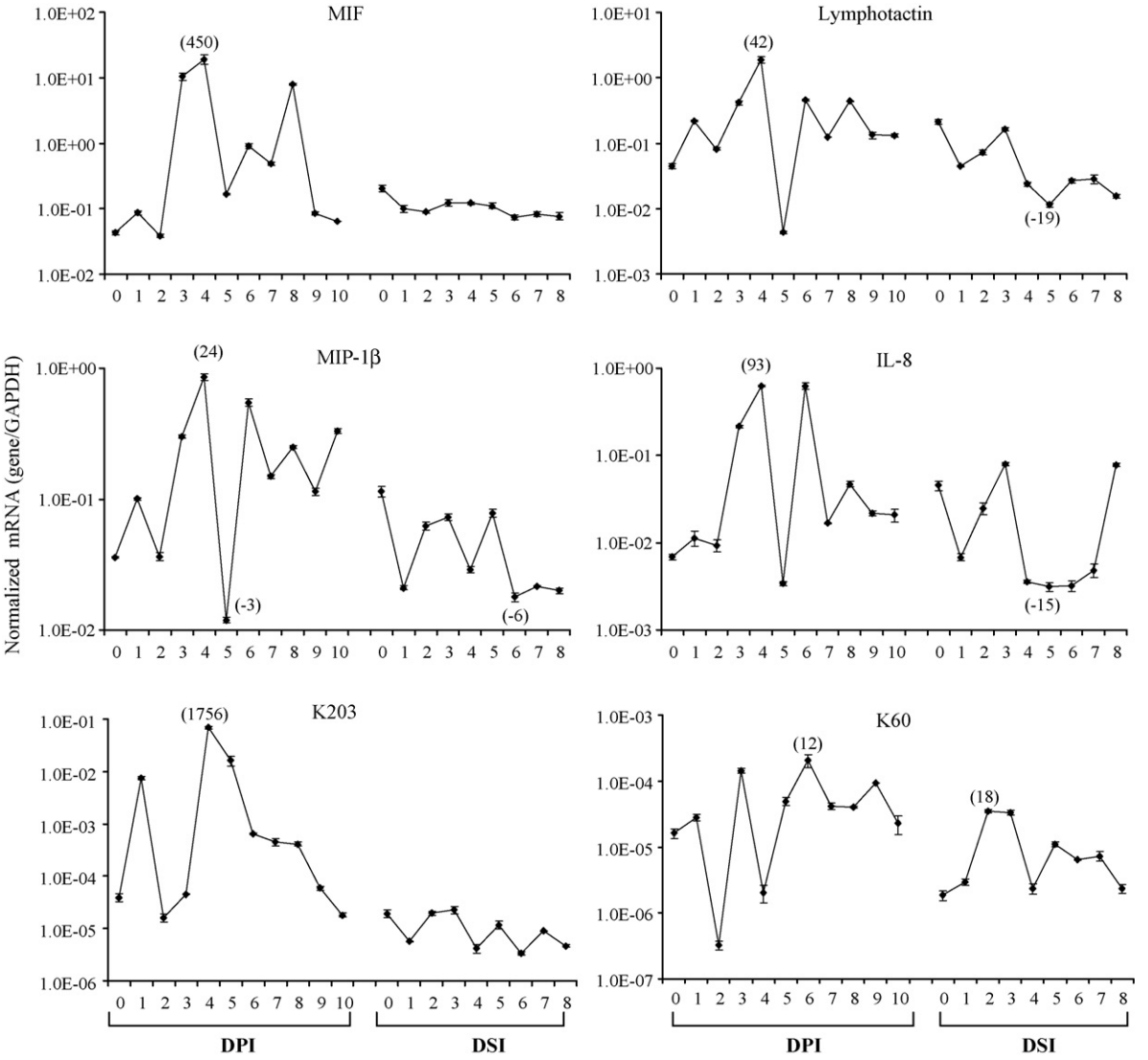

Fig. 4. Quantitative RT-PCR determination of chemokine mRNA levels after *E. maxima* infection. Chickens were infected with *E. maxima* and mRNA levels determined as in Fig. 1.

2000). Our current results showed that IL-15 mRNA expression was higher than IL-2 (Fig. 2) during the early innate immune response, suggesting that the function of IL-15 may be replaced by IL-2 after the adaptive immune response develops.

In addition, we investigated the expression of several other genes encoding molecules that are associated with Th1 responses. T-bet, an important Th1-driving transcription factor, plays a major role in regulating IFN-γ production in various cell types leading to Th1 cell migration to sites of inflammation (Lord et al., 2005; Ylikoski et al., 2005). T-bet and GATA binding protein-3 (GATA-3) also are important regulators of Th1 ($CD4^+$) and Th2 ($CD8^+$) differentiation that are activated through the IL-12/STAT4 and IL-4/STAT6 pathways (Hwang et al., 2005). T-bet is expressed in $CD4^+$ and $CD8^+$ T cells, NK cells, and dentritic cells, and T-bet deficient cells failed to differentiate along the Th1 pathway (Ylikoski et al., 2005). T-bet was required for development of resistance to *Salmonella* infection (Ravindran et al., 2005). Our own studies showed that T-bet mRNA expression was augmented in the $CD4^+$ and $CD8^+$ T cells following *E. acervulina* infection (unpublished observations).

IFN-γ is a potent iNOS inducer (MacMicking et al., 1997). Thus, the high levels of IFN-γ and iNOS transcripts seen in the mucosa of *E. maxima*-infected animals are probably related, as reported in the case of

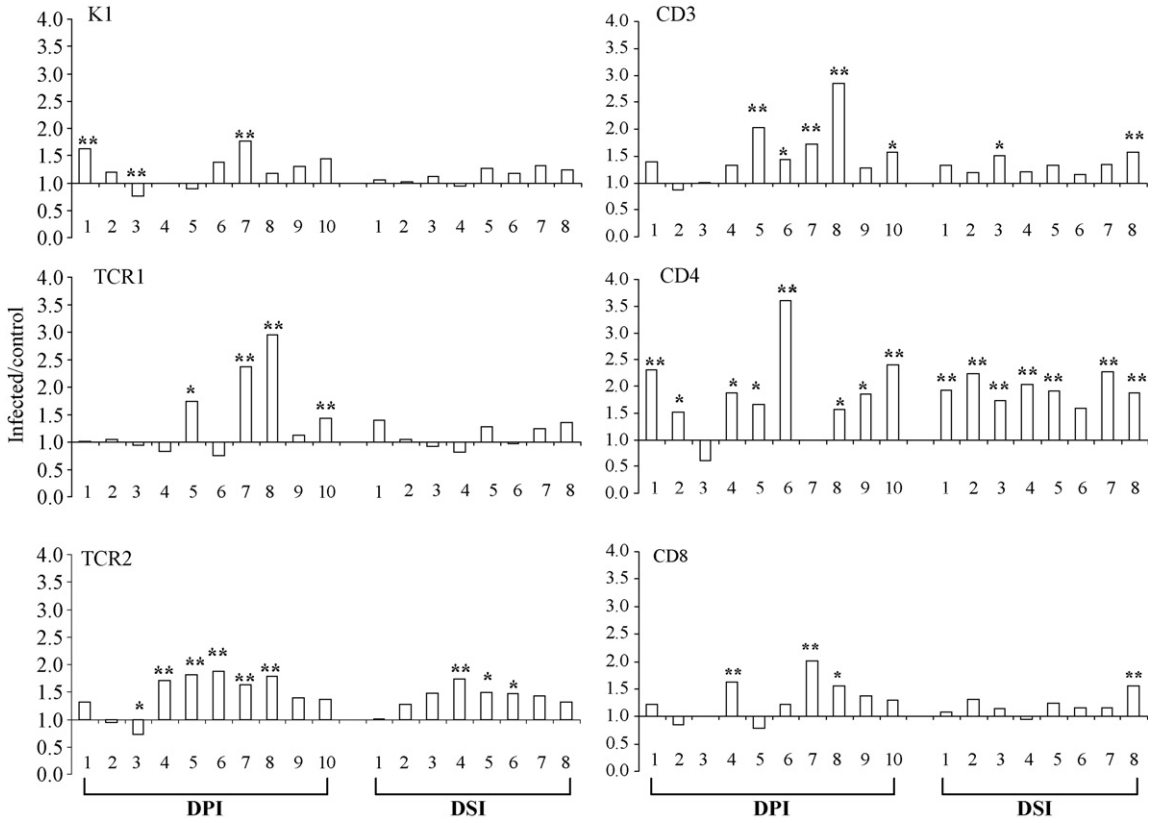

Fig. 5. Subpopulations of intestinal IELs following infection with *E. maxima*. Chickens were infected with *E. maxima* as described in Fig. 1, and IELs were analyzed by flow cytometry using mAb against the following surface markers: K1, a chicken macrophage marker, CD3, TCR1 (γδ-TCR), CD4, TCR2 (αβ-TCR), and CD8. The percentages of cells recognized by the mAbs were calculated with respect to those bound by the C6B12 mAb after normalized to bind with normal IgG. Asterisks indicate significantly increased level of cells when comparing with age-matched non-infected controls ($^{*}p < 0.01$; $^{**}p < 0.001$). DPI, days post-primary infection; DSI, days post-secondary infection.

Salmonella infection (Sadeyen et al., 2004). Previous studies showed that during *E. maxima* infection, the levels of nitrite and nitrate reached peak values at day 6 post-inoculation (Allen, 1997). Similarly, our current data showed an initial peak in iNOS mRNA at day 4 and a second peak at day 6 post-infection.

MyD88 and TRAF-5 are adaptor molecules essential for signal transduction during Th1 responses. MyD88 is central to signaling by most TLRs as well as by IL-1 and IL-18, while TRAF-5 is involved in CD30 signaling (Aizawa et al., 1997). MyD88$^{-/-}$ mice are acutely susceptible to a wide variety of bacterial, fungal, protozoan, and viral infections (Takeda et al., 2003). By contrast, our results suggested that expression of these molecules were not greatly affected by *E. maxima* infection.

The chicken Th2 cytokine gene cluster was the first to be characterized outside of mammals and contains homologues of IL-3, IL-4, IL-13, and GM-CSF. Compared with mammalian IL-3, chicken IL-3 was reported to be expressed at higher levels in all tissues (Avery et al., 2004), which concurs with our mRNA expression data. IL-13 is structurally similar to IL-4 and is produced by Th2 CD4^{+} T cells as well as epithelial cells. When infected with *Ascaridia galli* worm eggs, chicken IL-13 mRNA expression was significantly augmented at day 14 post-infection (Degen et al., 2005). Similarly, we observed increased IL-13 mRNA expression level following secondary *Eimeria* infection (Fig. 3). GM-CSF is a cytokine generally made by activated both type of T cells (Th1 and Th2), macrophages, endothelial cells, and fibroblasts. It promotes the maturation of bone marrow cells into dendritic cells and monocytes. Functionally, chicken GM-CSF was shown to inhibit the synthesis of pro-inflammatory cytokines (including IL-1β, TNF-α, and IL-12), thus down-regulating inflammatory (Th1) responses (de Waal Malefyt et al., 1991; Groux and Powrie, 1999), which concurs with our results presented in Figs. 1 and 2.

TGF-β is an important regulator of inflammation, exhibiting pro-inflammatory properties at low concentrations and anti-inflammatory effects at high concentrations (Omer et al., 2000). Simultaneous administration of chicken TGF-β4 or IL-1β genes with a cDNA encoding the 3-1E protein vaccine reduced fecal oocyst shedding, but these cytokines had no effect on IEL subpopulation dynamics (Min et al., 2001). Our result describing little changes of TGF-β4 mRNA following *E. maxima* infection is consistent with these and other studies (Jakowlew et al., 1997; Choi et al., 1999; Laurent et al., 2001).

In general, the C and CC chemokines are involved in the recruitment of macrophages and lymphocytes respectively, whereas CXC chemokines participate in the recruitment of neutrophils to sites of inflammation. Laurent et al. (2001) showed that mRNA levels of the CC chemokines MIP-1β and K203 were up-regulated 200- and 80-fold, respectively, in the ceca in response to *E. tenella* infection, and 100- and 5-fold in the jejunum following *E. maxima* infection. Although we also observed that expression of both transcripts was increased following infection with the same organism, the relative levels were inverted, i.e., K203 transcripts were up-regulated to a greater extent than those of MIP-1β. The CXC chemokines IL-8 and K60 are also produced in the gut and their mRNA expression patterns were similar following parasite primary infection as well as *Salmonella* infection indicating that they may play a key role in the initiation of inflammation (Laurent et al., 2001; Swaggerty et al., 2004; Withanage et al., 2004).

A unique feature of chicken intestinal IELs is that γδ-TCR ($TCR1^+$) cells constitute the predominant population of T cells (Lillehoj, 1994). In previous reports, we documented that infection of chickens with *E. acervulina* or *E. tenella* increased the numbers of $TCR1^+$ IELs in the intestine (Lillehoj, 1994; Yun et al., 2000a). In another study, during *E. maxima* infection, both $CD4^+$ and $CD8^+$ cells were observed by immunohistochemistry in the small intestine of Light Sussex chickens, but the proportion of $CD8^+$ cell was higher than that of $CD4^+$ cells (Rothwell et al., 1995). The current study now extends these observations to *E. maxima* infected animals at various time-points. Although $CD4^+$ cells represent a minor subset of chicken intestinal IELs, our results showed that $CD4^+$ IELs were increased at days 5–6 following primary infection with *E. maxima*, which is similar to that reported after infection with *E. acervulina* (Lillehoj, 1994; Bessay et al., 1996) and *E. tenella* infections (Bessay et al., 1996; Yun et al., 2000a). In particular, the $CD4^+$ IELs cell ratio remained elevated following secondary infection which means a small subset of $CD4^+$ cells may be responsible for resistance to coccidiosis as shown in the case of *Cryptosporidium muris* (McDonald et al., 1994). On the contrary, the $CD8^+$ and $TCR1^+$ cells had no differences between infected and non-infected controls at secondary infection indicating that a protective host immune response, cell- or antibody-mediated, had developed subsequent to primary infection.

It is also noteworthy to mention that the day-to-day mRNA variation during *E. maxima* infection was more pronounced compared with *E. acervulina* or *E. tenella* infections (Y.H. Hong, unpublished data). Inflammatory cytokines and chemokines were momentarily up-regulated at day 1 following primary infection and then showed biphasic kinetics at days 3 or 4, and 6. Nonetheless, all cytokine mRNA levels were down-regulated at day 5 following primary infection which concurs with reductions in macrophages and $CD8^+$ cells at the same day (Fig. 5).

In conclusion, the results presented herein characterize the dynamics of cytokine/chemokine gene specific responses associated with *E. maxima* infection. During avian coccidiosis, both types of immune effector molecules play key roles in the protective response to *Eimeria* infection, although it is important to note that mRNA levels documented in this report may not correlate with the amount of active protein expressed *in vivo*. In addition to cytokines and chemokines, intestinal lymphocyte subpopulations regulate *Eimeria*-induced inflammation such that clearance of primary parasite infection is dominated by Th1 responses. Finally, these results also provide a possible rationale for use for cytokines and chemokines as therapeutic agents against coccidiosis (Lowenthal et al., 1997; Lillehoj and Choi, 1998; Min et al., 2001).

Acknowledgments

We thank Dong Woon Park, Margie Nichols and Diane Hawkins-Cooper for animal studies and Rami A. Dalloul for critical review. This project was supported, in part, by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, grant # 2004-35204-14798.

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